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Methods in Enzymology

Volume 186

Oxygen Radicals in Biological Systems

Part B

Oxygen Radicals and Antioxidants

EDITED BY

Lester Packer
Alexander N. Glazer

DEPARTMENT OF MOLECULAR AND CELL BIOLOGY
UNIVERSITY OF CALIFORNIA, BERKELEY
BERKELEY, CALIFORNIA

Editorial Advisory Board

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego New York Boston
London Sydney Tokyo Toronto

This book is printed on acid-free paper.

ACADEMIC PRESS, INC.
San Diego, California 92101

United Kingdom Edition published by
ACADEMIC PRESS LIMITED
24-28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 54-9110

ISBN 0-12-182087-4 (alk. paper)

PRINTED IN THE UNITED STATES OF AMERICA
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plasma.[58] The details of this GC–MS method are described elsewhere in this volume [40, 41].

Concluding Remarks

The choice of which method for aldehyde analysis should be used depends on the particular interest of the investigator. Is an overall picture of the complete spectrum of aldehydes required, or is there an interest in a specific compound such as MDA or HNE? If only MDA is to be determined, the classic TBA test remains a useful method, providing it has been validated by an HPLC measurement for the particular system under study. If only HNE is to be determined, the method of choice is direct HPLC or GC–MS. The latter method is more sensitive, but the resources required are more expensive.

If the whole spectrum of aldehydes must be measured, then the DNPH method described is probably more reliable than the current cyclohexanedione method, which separates all aldehydes in one run. As the number of aldehydes present in peroxidized biological samples may exceed 30 and their relative proportions vary greatly, complex chromatograms are produced and definite peak identification is difficult. The DNPH method is less sensitive but gives more confidence in peak identification.

Acknowledgments

The authors' work has been supported by the Association for International Cancer Research (U.K.) and by the Austrian Science Foundation (to H.E., Project P6176B).

[58] M. L. Selley, M. R. Bartlett, J. A. McGuiness, A. J. Hapel, and N. G. Ardlie, *J. Chromatogr.* **488**, 329 (1989).

[43] Malondialdehyde Determination as Index of Lipid Peroxidation

By H. H. Draper and M. Hadley

Introduction

The determination of malondialdehyde (MDA) has attracted widespread interest because it appears to offer a facile means of assessing lipid peroxidation in biological materials. However, the validity of MDA as an index of lipid peroxidation has been clouded by controversy regarding its formation as an artifact of analysis and as a product of enzyme reactions,

its occurrence in various bound forms, and the specificity of methods used for its measurement. Some investigators, on the basis of evidence that MDA fails to reflect extensive peroxidation in certain biological materials, have questioned its validity as an index of peroxidation in all such materials. Other investigators have found that MDA is a reliable indicator of peroxidation in many samples, and that difficulties in its determination can be resolved by appropriate modifications in methodology.

Malondialdehyde occurs in biological materials in the free state and in various covalently bound forms. In the only materials so far extensively examined (food and urine), it has been found predominantly in bound forms. Most of the MDA in foods of animal origin (~80%) appears to be bound in Schiff base linkages to the ε-amino groups of the lysine residues of food proteins, from which it is released during digestion as *N*-ε-propenallysine.[1] A portion of the remaining MDA is present in the form of other minor derivatives. *N*-ε-Propenallysine and its N-α-acetylated derivative are the main forms in which endogenous MDA is excreted in rat and human urine.[2,3] Urine also contains small amounts of MDA adducts with guanine, the phospholipid bases serine and ethanolamine, and other unidentified reactants. Free MDA is a minor and variable excretory product.

It is apparent from the occurrence of these derivatives in urine that MDA forms adducts with proteins, nucleic acids, and other substances *in vivo*, and this compromises the assessment of lipid peroxidation in the tissues based on the determination of free MDA. For example, although no free MDA is detectable in human plasma,[4] it is found using procedures that involve hydrolysis of bound forms.[5] Free MDA reacts extensively with the serum albumin present in cell culture media,[6] and may do the same in plasma. It may also react with proteins *in vitro*, particularly over long incubation periods.

MDA can be released from its bound forms by hot acid or alkali digestion, but the conditions required for hydrolysis can lead to other complications. The pH required for maximum yield of MDA varies among biological materials depending on the nature of the derivatives present. MDA may be generated during hydrolysis by oxidation of polyunsatu-

[1] L. A. Piché, P. D. Cole, M. Hadley, R. van den Bergh, and H. H. Draper, *Carcinogenesis* 9, 473 (1988).
[2] L. G. McGirr, M. Hadley, and H. H. Draper, *J. Biol. Chem.* 260, 15427 (1985).
[3] H. H. Draper, M. Hadley, L. Lissemore, N. M. Laing, and P. D. Cole, *Lipids* 23, 620 (1988).
[4] C. Largillière and S. B. Mélancon, *Anal. Biochem.* 170, 123 (1988).
[5] M. A. Warso and W. E. M. Lands, *Clin. Physiol. Biochem.* 2, 70 (1984).
[6] R. P. Bird and H. H. Draper, *Lipids* 17, 519 (1982).

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rated fatty acids (PUFA) in the sample and by degradation of preexisting oxidation products. Pigments present in the sample, or generated during hydrolysis, also can interfere in the colorimetric assessment of MDA. These problems, and possibilities for their resolution, are discussed in the following sections.

Absorptiometric Methods

Although absorptiometry is the method which has been most frequently used to quantify MDA, many different procedures have been used for its extraction from the sample and for its purification, depending on the nature of the material being analyzed and whether free or total MDA is estimated.

Free MDA. Below its pK_a of 4.65, MDA exists primarily in a cyclic form with a UV_{max} at 245 nm (E_{mol} 1.37×10^4).[7] Raising the pH above 4.65 results in increasing proportions of the enolate ion, which has a UV_{max} at 267 nm (E_{mol} 3.42×10^4).[8] At neutral pH the enolate anion is the predominant form.

Esterbauer and Slater[7] determined free MDA in incubates of peroxidizing hepatic microsomes by measuring its UV absorbance after isolation using high-performance liquid chromatography (HPLC). Aliquots of homogenate were applied to a column designed for carbohydrate analysis, and MDA was measured in the eluates at 270 nm using an absorbance detector. The method gave values similar to those obtained by an unspecified 2-thiobarbituric acid (TBA) procedure, indicating that the MDA formed was still in the free state at the end of the 30-min incubation period and that there was no interference by other aldehydes in the TBA method for MDA determination.

This procedure is short (reported time of analysis <10 min), sensitive (5 ng on the chromatogram), and apparently specific for MDA. It should be useful in monitoring lipid peroxidation *in vitro* over short time periods, and in studying such phenomena as radiation-induced hydroxyl radical degradation of sugars,[9] but it is not suitable for the analysis of complex biological materials, such as food and urine, in which most of the MDA exists in the form of derivatives. For the same reason, it probably has limited application to animal and human tissues.

Largillière and Mélancon[4] applied a modification of the procedure to the determination of MDA in deproteinized human blood plasma. They

[7] H. Esterbauer and T. F. Slater, *IRCS Med. Sci.* **9**, 749 (1981).
[8] L. J. Marnett, M. J. Bienkowski, M. Raban, and M. A. Tuttle, *Anal. Biochem.* **99**, 458 (1979).
[9] K. H. Cheeseman, A. Beavis, and H. Esterbauer, *Biochem. J.* **252**, 649 (1988).

failed to detect any free MDA and concluded that "the classical thiobarbituric acid test" is not suitable for determination of MDA in plasma. However, if (as the authors speculate) MDA in plasma is present in complexes with proteins and amino acids, from which it is released by acid hydrolysis under the conditions of the TBA reaction, the converse conclusion may be drawn (i.e., that procedures for the determination of free MDA are not suitable for the analysis of plasma).

Lee and Csallany estimated free MDA in an ultrafiltrate of rat liver homogenate from its absorbance at 267 nm after purification by HPLC on a size-exclusion column.[10] The levels found in the liver of vitamin E-deficient animals were about 15 times those found in control livers. Bound MDA was estimated by subtracting values for free MDA from those obtained after hydrolysis at pH 13 for 30 min at 60°. The concentration of bound MDA was increased only 2-fold in deficient liver, and its concentration in normal liver exceeded that of free MDA. Although the results suggest that free MDA accumulates in the liver in vitamin E deficiency, it is possible that more free MDA was formed in the deficient tissue during the analytical procedure, which did not include the use of a synthetic antioxidant. Analysis for TBA-reactive substances, carried out on a butanol extract of the reaction mixture using a conventional spectrophotometric procedure,[11] yielded values that were 1.4–1.8 times higher than those for total MDA.

Bull and Marnett[12] used myristyltrimethylammonium bromide to prevent polymerization of MDA during HPLC. Chromatography on a reverse-phase column using a mixture of acetonitrile and this ion-pairing reagent enabled MDA to be selectively eluted as the conjugate base and quantified from its absorbance at 267 nm. The procedure was used successfully to estimate free MDA production in peroxidizing liver microsomes and to demonstrate that MDA in urine is present in the form of metabolites. Values similar to those obtained using the colorimetric TBA procedure[11] were observed at high concentrations of MDA in the microsomes, but lower values were found at low concentrations, indicating that the TBA method was nonspecific for MDA (or that MDA was released from bound forms during the TBA procedure).

MDA Derivatives. MDA reacts with a variety of compounds to form derivatives which can be estimated from their absorption in the visible region. These include aniline, 4-hexylresorcinol, *N*-methylpyrrole, indole, 4-aminoacetophenone, ethyl *p*-aminobenzoate, 4,4-sulfonyldiani-

[10] H. Lee and A. S. Csallany, *Lipids* **22**, 104 (1987).
[11] R. O. Sinnhuber, J. C. Yu, and T. C. Yu, *Food Res.* **23**, 620 (1958).
[12] A. W. Bull and L. J. Marnett, *Anal. Biochem.* **149**, 284 (1985).

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line, *p*-nitroaniline, and azulene.[13] Other aldehydes also react with most of these compounds to form yellow or orange complexes which can interfere in the estimation of MDA if they are present in the sample and are not separated from the MDA complex.

The most widely employed method for the determination of MDA in biological materials is based on its reaction with TBA to form a pink complex with an absorption maximum at 532–535 nm (E_{mol} 14.9×10^4).[7] Heating the sample at a pH of 3 or below is necessary for complex formation and for release of MDA from bound forms. The classic procedure of Sinnhuber *et al.*[11] (often referred to in the literature as "the TBA procedure") involves heating a trichloroacetic acid (TCA) extract of the sample with TBA and measuring the absorbance of the crude mixture in a spectrophotometer.

This simple procedure is subject to several sources of error. The apparent MDA content of many materials of plant and animal origin is inflated by pigments which absorb in the same region as the TBA–MDA complex. On the other hand, MDA content may be underestimated as a result of adsorption of the TBA complex onto the protein precipitate. Other aldehydes, if present, can react with TBA to produce a colored complex. Also, MDA may be formed during the procedure by oxidation of PUFA and by decomposition of oxidized lipids in the sample.

Oxidation of PUFA can be extensively reduced, if not eliminated, by adding butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) to the reaction mixture before processing,[14] and this precaution should be an integral part of the TBA procedure. Chelators may either inhibit or increase metal catalysis of lipid peroxidation. For example, EDTA may promote the formation of hydroxyl radicals, which catalyze lipid peroxidation, by maintaining Fe^{3+} in a soluble form, whereas desferrioxamine may inhibit their formation by blocking the reduction of Fe^{3+} to Fe^{2+}.[15] However, in a study on the MDA content of fish meal, EDTA ($\geq 25\ \mu M$) added to the TBA reaction mixture reduced the value obtained by 22% and desferrioxamine (100 μM) by 48% (H. H. Draper, M. Hadley, and A. J. Ninacs, unpublished).

Formation of MDA by decomposition of preformed lipoxides during the procedure is a more intractable problem, the significance of which depends on the nature of the sample and the purpose of the analysis (i.e., whether assessment of lipid peroxidation or of MDA specifically is the objective). In some materials, such as urine, the quantity of oxidizable

[13] E. Sawicki, T. W. Stanley, and H. Johnson, *Anal. Chem.* **35**, 199 (1963).
[14] J. Pikul, D. E. Leszczynski, and F. A. Kummerow, *J. Agric. Food Chem.* **31**, 1338 (1983).
[15] B. Halliwell and J. M. C. Gutteridge, *Arch. Biochem. Biophys.* **246**, 501 (1986).

lipids present is negligible, whereas in food and tissue samples they may contribute significantly to the amount of MDA determined as the TBA complex. In thermally oxidized oils there may be little relationship between the amount of MDA found using the colorimetric procedure[11] and that found using a gas chromatography–mass spectrometry method specific for MDA.[16] The main precursors of MDA generated in such oils under the conditions required for TBA–MDA complex formation are five-membered hydroperoxy epidioxides and 1,3-dihydroperoxides.[16]

The contribution of oxidized lipids in food and tissue samples to MDA determined as the TBA derivative cannot be determined using current methodology and undoubtedly is highly variable. When analyzing such samples for MDA using a TBA procedure, any interference by pigments and other TBA-reactive substances should be removed and lipid peroxidation reported in terms of MDA equivalents. For most such samples, the error arising from decomposition of oxidized lipids is likely to be much smaller than the error arising from failure to release MDA from its bound forms. Further, inclusion of MDA generated by decomposition of oxidized lipids during the TBA procedure may provide a better assessment of lipid peroxidation in the sample.

MDA can be formed from some sugars, including sucrose and 2-deoxyribose, by hydroxyl radical-generating procedures such as γ-radiolysis of water and incubation with a high concentration of ferrous ions.[9] However, these conditions are unlikely to occur in biological materials. It has been found, for example, that when 0.25 *M* sucrose was used to prepare mitochondria, the absorbance of the sample blank was not significantly different from that of a water blank.[17]

Interference by other TBA reactants and by pigments can be overcome by isolating the TBA–MDA complex using HPLC prior to determining its absorbance.[18] In the case of urine, in which such interference (as well as the concentration of oxidized lipids) is negligible, the amount of TBA–MDA complex found using an HPLC purification procedure[19] is similar to that found by measuring the absorbance of the crude reaction mixture at 532 nm.[11] In the case of tissue and food samples, which normally contain oxidized lipids, separation of the TBA–MDA complex usually results in lower values.[18]

The fact that aldehydic compounds other than MDA can react with TBA to form a complex that absorbs in the 532–535 nm region has led to use of the term thiobarbituric acid-reactive substances (TBA-RS), ex-

[16] E. N. Frankel and W. E. Neff, *Biochim. Biophys. Acta* **754**, 264 (1983).
[17] G. M. Siu and H. H. Draper, *Lipids* **17**, 349 (1982).
[18] R. P. Bird, S. S. O. Hung, M. Hadley, and H. H. Draper, *Anal. Biochem.* **128**, 240 (1983).
[19] H. H. Draper, L. Polensek, M. Hadley, and L. G. McGirr, *Lipids* **19**, 836 (1984).

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pressed in MDA equivalents, to characterize the products of the TBA reaction quantified using the conventional spectrophotometric method.[11] Considering the frequency of references to such derivatives in the literature, there is a surprising paucity of evidence for their actual existence in biological materials. The higher MDA values for tissue samples obtained using the colorimetric TBA procedure,[11] as opposed to a procedure for free MDA, are often attributed to TBA reactions with other aldehydes, but they are more likely to be due to release of bound MDA by acid hydrolysis and/or the presence of pigments in the sample. In any event, isolation of the TBA–MDA complex should be a standard part of the TBA method for MDA determination unless it has been found to be unnecessary in the case of specific samples.

In the TCA extracts of samples high in protein, such as animal tissues, the TBA–MDA complex may adsorb onto the protein precipitate, imparting to it a pink color. This can be largely avoided by removing the protein precipitate before carrying out the TBA reaction. However, some TBA–MDA complex may be bound to peptides soluble in TCA and may be lost in procedures used to purify the complex prior to its estimation. For example, purification of the crude TBA reaction mixture on a Sep-Pak C_{18} cartridge[18] leaves a reddish residue on the cartridge after elution of the free TBA–MDA complex with methanol. Investigation of this so-called TBA-RS material has revealed that it is, in fact, TBA–MDA complex bound to residual protein in the TCA extract. Removal of this bound complex accounts in large part for the lower MDA values obtained by isolating the free TBA–MDA complex[18] and those obtained by spectrophotometric measurement of the free and bound complex in the crude reaction mixture.[11] No evidence of TBA-RS, other than TBA–MDA could be found in human blood serum, pig liver, or fish meal (H. H. Draper, M. Hadley, and A. J. Ninacs, unpublished).

MDA can be determined unambiguously as the TBA–MDA complex using HPLC, and it is therefore not appropriate to report values obtained by this procedure as TBA-RS. It should be recognized, however, that the value obtained by this method may reflect some combination of free and bound MDA originally present in the sample plus MDA formed from oxidized lipids during the procedure. Hence, the method provides a measure of lipid peroxidation in the sample in terms of MDA equivalents. Whether MDA formed by decomposition of oxidized lipids during the procedure is an "artifact" is largely a matter of perception. Since all MDA in a sample (except that of enzymatic origin) is a secondary product of lipid peroxidation, any distinction between MDA formed from oxidized lipids prior to analysis and MDA formed from oxidized lipids during analysis may be fallacious.

HPLC Procedures for Total MDA

The following procedures have been found satisfactory for the determination of total MDA in food samples, animal tissues, and urine.

HPLC Conditions. A 0.39 × 30 cm μBondapak C_{18} stainless steel analytical column attached to a 3 × 22 mm guard column packed with C_{18}/Corasil (Waters, Milford, MA) is used. The absorbance detector is equipped with a 546 nm interference filter attached to a data module for integration of peaks and printout of the elution profile. Sensitivity is set at 0.005 AUFS. The mobile phase is 18% HPLC-grade methanol in distilled water degassed by filtering through a 0.45-μm filter under vacuum with constant stirring. A flow rate of 2.0 ml/min is controlled by a two-phase solvent pump.

Standard Curve. A standard curve is prepared using TBA–MDA complex which has been checked for purity by HPLC, NMR, and elemental analysis. Instrument response is plotted against the molar equivalent weight of MDA in the complex injected. Instrument sensitivity is 1 ng of TBA–MDA complex.

Determination of MDA in Foods and Tissue Samples. The sample (0.5–1.0 g) is homogenized in 5 ml of 5% aqueous TCA plus 0.5 ml of methanolic BHT (0.5 g/liter) and heated in a capped tube for 30 min in a boiling water bath to release protein-bound MDA. To avoid adsorption of the TBA–MDA complex onto insoluble protein, any solid particulate material observed after cooling to room temperature is removed by centrifugation at 1000 *g* for 10 min. A 1-ml aliquot of the supernatant (in duplicate) plus 1 ml of a saturated solution of TBA reagent are heated in boiling water at pH 1.5 for 30 min. After cooling, an acceptable estimate of the MDA content of some samples, including urine, can be obtained by measuring the absorbance of the crude mixture at 532 nm using a spectrophotometer. Two duplicated blanks are used: a reagent blank to zero the instrument and a sample blank that is subtracted from the reading for the sample.

To separate TBA–MDA from other possible TBA–RS, the cooled sample is loaded onto a Sep-Pak C_{18} cartridge (Waters) that has been prewashed with 15 ml of methanol followed by 15 ml of distilled water. The column is developed with 4 ml of distilled water, which is discarded, then with 2 ml of methanol, which is collected in a 4-ml vial. The methanol is evaporated at 70° on a sand bath using a stream of air. The residue is dissolved in water (usually 1 ml) and an aliquot (usually 10–20 μl) is injected onto the HPLC column. Two reagent blanks in duplicate are used. Sample blanks have been found unnecessary. In the case of samples high in protein, this procedure may underestimate MDA content as a

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Determination of MDA in Urine. One milliliter of rat or human urine is heated at pH 3.0 ± 0.1 with 4 ml of saturated TBA reagent in a boiling water bath for 30 min. For most purposes, MDA can be satisfactorily estimated from the absorbance of TBA–MDA complex in the cooled sample at 532 nm using a spectrophotometer as described above.

To determine MDA specifically, the cooled sample is subjected to purification on a Sep-Pak cartridge in the manner described for food and tissue samples. After removing the methanol, the residue is dissolved in 2.0 ml of double-distilled water, and an aliquot (10–200 μl) is injected onto the HPLC column. The concentration of MDA in rat urine is typically about 5 times that in human urine. A sample blank and a TBA blank are carried through the procedure in duplicate.

Notes Regarding Procedures

1. The methods described have been found to be specific for MDA and to have good reproducibility. The method for food and tissue samples generally yields lower values than the colorimetric TBA procedure,[11] whereas for urine the values are generally similar.
2. Sep-Pak cartridges can be reused 3–10 times by subjecting them to the pretreatment procedure between samples.
3. In the case of samples that contain large amounts of MDA, more than 2 ml of methanol may be necessary to elute the TBA–MDA complex from the Sep-Pak. Elution of the pink complex can be monitored visually.
4. The TBA–MDA complex should be applied to the HPLC column in water. When applied in methanol, the complex often yields two peaks, one at the solvent front and one at the R_t found with water. The solvent front peak reverts to the proper R_t when it is reinjected in water.
5. The procedure for foods and tissues probably can be applied, with minor modifications, to plasma, cell fractions, *in vitro* incubation mixtures, and other tissue samples.
6. The HPLC column should be rigorously cleaned after 30–40 samples have been injected.
7. Some urine samples form a precipitate, to which the TBA–MDA complex adsorbs, during the procedure. This can be prevented by

heating the urine in a capped tube for 30 min in a boiling water bath at pH 3.0 ± 0.1 in the absence of TBA reagent, then removing the precipitate by centrifugation and using the supernatant fraction for MDA analysis.

8. Variations may be observed in the R_t of the TBA–MDA complex for the same sample. These variations usually can be eliminated by buffering the mobile phase.
9. During HPLC of some samples a "matrix effect" is observed, i.e., the TBA–MDA complex in the sample has a different R_t from that of the TBA–MDA standard. The peak for the sample can be identified by spiking with the standard, which migrates to the R_t of the complex in the sample.
10. In urinalysis for MDA, time can be saved by preparing the TBA reagent in 1.3 *M* phosphate buffer (pH 2.8), thereby avoiding the necessity of adjusting the pH of each sample. Otherwise, in the analysis of some samples, the pH may rise during the TBA reaction because ammonia is released by hydrolysis of urea, thereby inhibiting TBA–MDA complex formation. Complex formation decreases markedly when the pH exceeds 3.0. Four milliliters of TBA reagent is necessary for maximum complex formation. MDA does not react with the amino groups of urea under the conditions of the TBA reaction.

Gas Chromatographic Methods

Gas chromatography (GC) procedures have been developed for determining free MDA in oils and fats after its conversion to appropriate derivatives. These procedures have been developed mainly to avoid the risk of MDA generation under the hot, acidic conditions required for formation of the TBA derivative. Free MDA in photoirradiated PUFA, corn oil, and beef fat has been determined by reacting it with methylhydrazine to form 1-methylpyrazole, which was measured using a nitrogen–phosphorus-specific detector and a fused silica capillary column.[20] A similar detector has been used to determine MDA in working solutions and in urine after derivitization using 2-hydrazinobenzothiazole.[21] MDA formed by decomposition of PUFA methyl esters has been determined by GC after conversion to a stable 1,3-dioxane derivative,[22] and MDA formed by decomposition of lipoxides has been measured after conversion to a stable tetramethylacetal derivative.[16]

[20] K. Umano, K. J. Dennis, and T. Shibamoto, *Lipids* **23**, 811 (1988).
[21] M. Beljean-Leymarie and E. Bruna, *Anal. Biochem.* **173**, 174 (1988).
[22] G. Lakshminarayana and D. G. Cornwell, *Lipids* **21**, 175 (1986).

So far, these methods
MDA in lipids. They hav
terials, in which MDA is

Other Methods

Fluorometric procedu
the older literature, but t
pounds, including 4,4-sul
benzoic acid, and 4-am
with MDA. The TBA–M
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pounds, as well as comp
derivatives. Kikugawa *et*
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reaction product 1,4-di
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A polarographic meth
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[23] K. Kikugawa, T. Kato, and
[24] A. M. Bond, P. P. Deprez, R
52, 2211 (1980).

[44] Cyclooxygenas

By RICHARD J
ROBERT B. PEN

Evaluation of the path
sensitive and specific ass
here exploits the ability o
ate the cyclooxygenase re
synthase. Because cyclo

for 30 min in a boiling water
of TBA reagent, then remov-
and using the supernatant

of the TBA–MDA complex
as usually can be eliminated

trix effect" is observed, i.e.,
e has a different R_f from that
for the sample can be iden-
ich migrates to the R_f of the

aved by preparing the TBA
2.8), thereby avoiding the
sample. Otherwise, in the
rise during the TBA reac-
hydrolysis of urea, thereby
on. Complex formation de-
ds 3.0. Four milliliters of
complex formation. MDA
urea under the conditions

been developed for deter-
rsion to appropriate deriv-
mainly to avoid the risk of
ns required for formation
lated PUFA, corn oil, and
methylhydrazine to form
a nitrogen–phosphorus-
mn.[20] A similar detector
lutions and in urine after
MDA formed by decom-
ined by GC after conver-
A formed by decomposi-
conversion to a stable

11 (1988).
174 (1988).
1986).

So far, these methods have been confined to the determination of free MDA in lipids. They have limited applicability to complex biological materials, in which MDA is present mainly in bound forms.

Other Methods

Fluorometric procedures for determining MDA have been reported in the older literature, but they have not found frequent use. Various compounds, including 4,4-sulfonyldianiline, ethyl *p*-aminobenzoate, *p*-aminobenzoic acid, and 4-aminoacetophenone, form fluorescent complexes with MDA. The TBA–MDA complex fluoresces with an emission maximum at 553 nm, but biological samples contain natural fluorescent compounds, as well as compounds other than MDA, that form fluorescent derivatives. Kikugawa *et al.*[23] have described a fluorometric method for the determination of free and acid-labile MDA in oxidized lipids as the reaction product 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde. These investigators found that their method yielded values lower than those obtained by the measurement of absorbance at 532 nm using the crude TBA reaction mixture, but they concluded that the latter method may provide a better index of lipid oxidation.

A polarographic method for determining MDA in 2 *M* HCl solution, with reported applicability to biological fluids, has been proposed.[24] This procedure, which measures free MDA (and possibly MDA present in some easily hydrolyzable derivatives), has not been extensively evaluated.

[23] K. Kikugawa, T. Kato, and A. Iwata, *Anal. Biochem.* 174, 512 (1988).
[24] A. M. Bond, P. P. Deprez, R. D. Jones, G. G. Wallace, and M. H. Briggs, *Anal. Chem.* 52, 2211 (1980).

[44] Cyclooxygenase Initiation Assay for Hydroperoxides

By RICHARD J. KULMACZ, JAMES F. MILLER, JR., ROBERT B. PENDLETON, and WILLIAM E. M. LANDS

Evaluation of the pathophysiological role of hydroperoxides requires a sensitive and specific assay for these compounds. The assay described here exploits the ability of low concentrations of hydroperoxides to initiate the cyclooxygenase reaction catalyzed by prostaglandin endoperoxide synthase. Because cyclooxygenase initiation requires only about 10^{-8} *M*